

AMENDMENTS TO THE SPECIFICATION:

Please replace the paragraph beginning at page 3, line 3, with the following rewritten paragraph:

--T lymphocytes obtained from tumor infiltrated lymph nodes were repeatedly stimulated in vitro with the autologous tumor and T cell clones were generated by limiting dilution. CD4+ T cells clones recognizing the autologous tumor in an HLA-DR restricted fashion were obtained, characterized in vitro for their fine specificity and used as cellular probe in a genetic approach aimed at defining the molecular nature of the recognized antigen. The screening of a cDNA expression library constructed using as template the RNA of the autologous melanoma led to the identification of the tyrosine phosphates receptor K gene (R-PTP-K) as encoding the antigen recognized by the CD4+ melanoma specific clones. The R-PTP-K mRNA cloned by melanoma cells contains a non-conservative Gly→Arg mutation in the fourth fibronectin III-like domain of the protein. This amino acid change generates a T cells epitope presented by the HLA-DRβ1*1001 that is recognized by the CD4 T cell clone used to screen the tumor cDNA library and by all the 5 different clones isolated from the tumor infiltrated lymph nodes of the same patient. The antigenic epitope was identified in the region 667-682 of ~~PTPRK~~_{Gly677→Arg682} PTPRK_{Gly677→Arg} and it has sequence PYYFAAELPPRNLPEP (SEQ ID NO: 1).--

Please replace the paragraph beginning at page 3, line 21, with the following rewritten paragraph:

-- A first aspect of the invention is directed to the immungenic peptide of SEQ ID NO: 1 and the use thereof in the generation of antibodies and/or T helper or cytotoxic cells, more generally in the induction of a tumor-specific immune response, for diagnostic or therapeutical applications, in particular for the diagnosis, prevention or immune therapy of tumors expressing ~~PTPRK_{Gly677→Arg682}~~ PTPRK_{Gly677→Arg}.--

Please replace the paragraph beginning at page 4, line 25, with the following rewritten paragraph:

--In a further embodiment, the invention provides polyclonal or monoclonal antibodies, fragments or derivatives thereof such as Fab, Fv or scFv, able to recognize and bind the peptide SEQ ID NO: 1. The isolated antibodies can be used in tumor immune therapy or in immune diagnostic techniques for the definition of tumors expressing ~~PTPRK_{Gly677→Arg682}~~ PTPRK_{Gly677→Arg}.--

Please replace the paragraph beginning at page 5, line 2, with the following rewritten paragraph:

-- In a yet further embodiment, the invention provides isolated CD4+ T cells specifically recognizing a tumor expressing ~~PTPRK_{Gly677→Arg682}~~ PTPRK_{Gly677→Arg} and the use thereof for

inducing a cell-mediate immune response against such tumor. These cells can be isolated from PBMC obtained from the patient to be subjected to the treatment, and they can be activated in vitro with the peptide SEQ ID NO: 1, optionally in the presence of cytokines, or using cells carrying the peptide in association with HLA-Class II molecules, such as APC (antigen presenting cells) expressing the allele HLA-DR β 1*1001 loaded with the peptide. APCs can be genetically modified, e.g. by transfection with a viral or retroviral vector, so as to express the specific allele HLA or the peptide or a precursor thereof. Modified HLA cells can be used to activate T cells either in vitro or in vivo. In vitro activated T cells can be subsequently reintroduced in the patient to prevent the onset, to arrest the growth or to reduce the amount of tumor cells. Before being reintroduced into the patient, lymphocytes may be purified, for example by means of an affinity column using an antibody directed against CD4 or other markers.--

Please replace the paragraph beginning at page 5, line 18, with the following rewritten paragraph:

--In a further embodiment the invention provides an isolated nucleic acid molecule encoding the epitope of ~~PTPRK_{Gly677→Arg682}~~ PTPRK_{Gly677→Arg} herein described, preferably the sequence

CCGTATTACTTTGCTGCAGAACTCCCCCGAGAAACCTACCTGAGCCT (SEQ ID N.
2)

as well as a vector and a host cell including said sequence. DNA molecules containing the peptide-encoding sequence, or a part thereof, and the gene constructs thereof can be used in the vaccination of subjects at risk of developing tumors, particularly melanoma, or cancer patients. DNA immunization can be carried out according to known techniques (Donnelly J.J. et al., 1994, The Immunologist 2:1). The intramuscular administration route is preferred, but also the parenteral and mucosal routes can be used (pnas 1986, 83, 9551; wo90/11092). Moreover, DNA can be adsorbed onto gold particles for the subcutaneous administration with a biolistic apparatus (Johnston, 1992 Nature, 356, 152).--

Please replace the paragraph beginning at page 6, line 5, with the following rewritten paragraph:

--In addition to the above described therapeutic uses, nucleic acid molecules containing the peptide-encoding sequence, or a part thereof, as well as the peptide itself, can be used in the diagnosis of melanoma expressing ~~PTPRK_{Gly677→Arg682}~~ PTPRK_{Gly677→Arg} for instance by PCR analysis or immunoassays using epitope-specific antibodies. Furthermore, complexes between the peptide SEQ ID NO: 1 and HLA-DR β 1*1001 cells can

be used for monitoring in vitro or ex vivo the immune response of subjects vaccinated with the peptide.--

Please replace the paragraph beginning at page 6, line 23, with the following rewritten paragraph:

-- **Figure 2**

Complementary DNA of clone #11 encodes for the TB515 recognized antigen. (A) CIITA⁺293 were transfected with cDNA#11 in pEAK8.5-Ii alone or together with pcDNA3.1-DRB1*0102 or pcDNA3.1-DRB1*1001, respectively. CIITA⁺293 singly transfected with pcDNA3.1-DRB1*0102 or pcDNA3-DRB1*1001, and CIITA⁺293 co-transfected with recombinant pcDNA3 encoding green fluorescent protein (GFP) and pcDNA3.1-DRB1*1001 were used as negative control. (B) cDNA#11 was subcloned in pcDNA3.1 and cotransfected with pcDNA3.1-DRB1*1001 in CIITA⁺293. Clone TB515 (1x10⁵ lymphocytes/well) was added to each transfectant and after 24 h, supernatants were collected and the content of IFN- γ evaluated by ELISA. In both panels, Me15392 was used as positive control. ~~Transfectats~~ Transfectants were all evaluated for the ability to induce IFN- γ release by TB515.--

Please replace the paragraph beginning at page 8, line 2, with the following rewritten paragraph:

-- **Figure 4**

Characterization of cDNA #11. cDNA #11 and related minigenes are represented as boxes aligned to a schematic structure of PTPRK protein. Black square in each minigene indicates the position of an ATG codon in frame with the starting ATG of the full-length gene (~~GenBank~~ GENBANKTM NM_002844). The mutated nucleotide (g→a) occurring at position 2249 is indicated. Minigenes were synthesized by PCR amplification of cDNA #11 using an identical forward primer (F2) coupled with different, nested reverse primers mapping downstream the mutation (EPR1, EPR2, EPR2WT, and EPR3 reverse primers, indicated by the arrows). Minigenes were cloned into expression vector pcDNA3/TOPO and then co-transfected with pcDNA3-DRB1*1001 or pcDNA3-DRB1*0102 into CIITA⁺-293 cells. Clone TB515 (1×10⁵ cells/well) was added to each transfectant, and after 24 h supernatants were evaluated for the content of IFN-γ by ELISA. In the table: +, positive recognition by TB515; -, no recognition by TB515. EP2wt minigene contained the non-mutated (g) nucleotide. Amino acid sequence in the bottom of the figure was deduced from the sequencing of cDNA #11. Abbreviations in the figure: LS, leader sequence; MAM, meprin/A5/R-PTPμ motif; Ig, immunoglobulin-like domain; FNIII, fibronectin type III-like domain; TM, transmembrane; PTP, protein-tyrosine phosphatase domain; R, arginine deriving from the nucleotide g→a mutation.--

Please replace the paragraph beginning at page 10,
line 13, with the following rewritten paragraph:

-- **MATERIALS AND METHODS**

Cell lines. The clinical course of patient (pt) 15392 (HLA-A*0301, B*40012, B*1402, C*0602, C*8002, DRB1*0102, DRB1*1001), and the in vitro stabilization of the melanoma cell line Mel15392 have been already described (16). By cell surface analysis Mel15392 cells were shown to be positive for class I HLA and to constitutively express DR, DP, but not DQ class II HLA. LCL15392 and LCL3700 are EBV-trasformed B cell lines obtained from peripheral blood mononuclear cells (PBMCs) of pt15392 and of an healthy donor, respectively. LCL3700 shares with pt15392 the DRB1*1001 only. 293-EBNA cells (wt293) (~~Invitrogen~~, INVITROGENTM, CA 9200, USA) and Class II Transactivator⁺ 293-EBNA cells (CIITA⁺293) were maintained in DMEM medium (Euroclone, Europe, TQ4 5ND Devon, UK) with 10% FCS. CIITA⁺293 cells were obtained by trasducing wt293 cells with CIITA-encoding retroviral vector. CIITA⁺293 cells were immunoselected using L243, a mAb specific for HLA-DR alleles.-

Please replace the paragraph beginning at page 13,
line 21, with the following rewritten paragraph:

-- **Northern Blot and RT-PCR analysis of R-PTPK expression.** Poly(A)+ RNAs from Mel15392, allogenic melanomas and PBL lines were isolated as described above. Total RNA

was isolated by using RNAqueous™-4PCR kit (~~Ambion~~, AMBION™, Austin, TX, 78744, USA). For Northern blot experiments, 10 µg of each RNA sample was subjected to electrophoresis in a 1 % formaldehyde agarose gel and transferred to a nylon membrane (Hybond-N+, ~~Amersham Biosciences~~, AMERSHAM BIOSCIENCES™, Inc. Piscataway, NJ 08855-1327, USA). The probes were labeled with [alpha-³²P]CTP by the random priming method (~~Amersham Biosciences~~ AMERSHAM BIOSCIENCES™), and pre-hybridization and hybridization were performed according to the Hybond-N+ paper guidelines. Membranes were washed four times with serially diluted solutions of SSC (from 0.03M to 0.0015M). Probes A and C were obtained by PCR amplification of Mel5392 poly(A)⁺ RNA with specific primers. Probe A, specific for the 5' region of the gene (bases 241-1110 of the gene), was synthesized with primers forward F1 (5'-GGCGCTGCCTGCTTTTGT-3'; SEQ ID NO: 16) and reverse R1 (5'-GGAGGAGCAATGGGTCTT-3'; SEQ ID NO: 17). Probe C, specific for the region encoding the two intracellular phosphatase domains (bases 2925-4547 of the gene), was derived with primers forward F3 (5'-CTTGGGATGTAGCTAAAAAAGATCAAAATA-3'; SEQ ID NO: 18) and reverse STOP (5'-CCAACTAAGATGATTCCAGGTACTCCAA-3'; SEQ ID NO: 19). All the amplification products were sequenced before being used as probes. DNA clone #11 (bases 2084-2751 of the gene) was used as probe B.--

Please replace the paragraph beginning at page 14, line 15, with the following rewritten paragraph:

-- The RT-PCR analyses of PTPRK expression-profile in normal and tumors cell lines were performed with the forward primer F3 and with the reverse primer Rδ (5'-CACCCTCTCTTTCAGCCAT-3'; SEQ ID NO: 20) under the following conditions: 2' 94°C, 34 cycles consisting of 1' 94°C, 2' 54°C, 3' 72°C, and finally 10' 72°C. Conditions were set in order to obtain linear DNA amplification. The amplified DNAs were loaded on agarose gels, stained with ethidium bromide, and analyzed with a dedicated software (Image Master VDL-CS, ~~Amersham Pharmacia Biosciences~~ AMERSHAM PHARMACIA BIOSCIENCES). Standard deviations were $\leq 5\%$ on triplicate experiments. The level of expression of each sample was normalized for RNA integrity by taking into account the level of expression of the β -actine gene (reaction conditions: 4' 94°C, 21 cycles consisting of 1' 94°C, 2' 68°C, 2' 72°C, and finally 10' 72°C, corresponding to linear DNA amplification).--

Please replace the paragraph beginning at page 14, line 28, with the following rewritten paragraph:

-- **Peptides synthesis.** Peptides were synthesized by conventional solid phase peptide synthesis, using Fmoc for transient NH₂-terminal protection, and characterized by mass spectrometry. All the peptides used were >95% pure

(~~Neosystem~~, NEOSYSTEM™, Strasbourg, France). Peptides were dissolved at 5 mg/mL in DMSO, stored at -20°C, and diluted in RPMI medium supplemented with 10 % human serum immediately before use.-

Please replace the paragraph beginning at page 15, line 6, with the following rewritten paragraph:

-- **Epitope reconstitution assay.** To analyze peptide recognition, 5×10^3 LCL15392 or LCL3700 cells were seeded in 96 microwells in 100 μ l of RPMI 1640-10% PHS and then pulsed with different concentrations of the relevant peptide. Peptide loading was allowed to proceed for 2 h at 37°C before effector cells were added to give a final E:T ratio of 1:1. Supernatants were collected after 18 h and IFN- γ content was determined by ELISA (~~Mabtech AB~~, MABTECH™, Stockholm, Sweden). Competition experiments were performed incubating 5×10^3 LCL15392 or 5×10^3 LCL3700 with various concentrations of the competitor peptides for 15 min before the addition of the PYYFAAELPPRNLPEP (SEQ ID NO: 1) peptide at 100nM. After 1 hr of additional incubation at 37°C, T cell clone was added at the final ratio of 1:1.-

Please replace the paragraph beginning at page 15, line 24, with the following rewritten paragraph:

-- IFN γ -Elispot assay. 96-well nitrocellulose plates (Millititer, ~~Millipore~~, MILLIPORETM, Bredford, MA) were coated overnight with 50 μ l/well of 8 μ g/ml anti-human IFN- γ mAb (~~Mabtech~~ MABTECHTM). Wells were then washed and blocked with Iscove's modified DMEM (~~BioWhittaker~~ BIOWHITTAKERTM) and 10% human AB-serum for 2 h at 37°C. T cells (2×10^3 or 2×10^4) were mixed with 1.5×10^4 peptide-pulsed autologous LCL cells and then seeded in the 96 pre-coated wells. T cells incubated with medium alone or with pokeweed mitogen served as negative and positive controls, respectively. After 24 h of incubation at 37°C and 5% CO₂, Elispot was then performed according to manufactory instructions. Briefly, plates were washed six times with PBS + 0.05% Tween-20. Wells were incubated for 2 h at 37°C with 50 μ l/well of biotinylated mouse anti-human IFN- γ mAb (~~Mabtech~~, MABTECHTM,) at a concentration of 2.5 μ g/ml. After washing four times with PBS, 100 μ l streptavidine-alkaline phosphatase (150 μ g/ml) diluted 1/1000 was added for 2 h at room temperature. After another washing step with PBS, 100 μ l/well of BCIP/NBT substrate (~~BioRad~~, BIORADTM, CA, 94547, USA) was added to each well for 10-20 min. Color development was stopped by washing under running tap water. After drying at room temperature, IFN γ secreting T cells were counted using the automated image analysis system Elispot

Reader (~~AID~~, AIDTM, Strassberg, Germany). Each experiment was performed in triplicate.--